Appendix A

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Expression and purification of a single-chain variable fragment antibody derived from a polyol-responsive monoclonal antibody

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Abstract

A previously described polyol-responsive monoclonal antibody (PR-mAb) was converted to a single-chain variable fragment (scFv). This antibody, PR-mAb NT73, reacts with the β′ subunit of *Escherichia coli* RNA polymerase and has been used for the immunoaffinity purification of polymerase. mRNAs encoding the variable regions of the heavy chain (V_H) and light chain (V_L) were used as the template for cDNA synthesis. The sequences were joined by the addition of a "linker" sequence and then cloned into several expression vectors. A variety of expression plasmids and *E. coli* hosts were used to determine the optimal expression system. Expression was highest with the pET22b(+) vector and the Rosetta(DE3)pLysS host strain, which produced approximately 60 mg purified His-tagged scFv per liter of culture (3.3 g wet weight cells). Although the production of soluble scFv was preferred, overproduced scFv formed inclusion bodies under every expression condition. Therefore, inclusion bodies had to be isolated, washed, solubilized, and refolded. The FoldIt protein refolding kit and enzyme-linked immunosorbent assay were sequentially used to determine the optimal refolding conditions that would produce active His-tagged scFv. Immobilized metal affinity chromatography was used for the final purification of the refolded active scFv. The polyol-responsiveness of the scFv was determined by an ELISA-elution assay. Although the scFv loses considerable affinity for its antigen, it maintains similar polyol-responsiveness as the parent monoclonal antibody, PR-mAb NT73.

Keywords: Single-chain variable fragment antibody; Polyol-responsive monoclonal antibody; *Escherichia coli* RNA polymerase; NT73; Inclusion body refolding

One DNA-dependent RNA polymerase accomplishes all transcription in *Escherichia coli.* Core RNA polymerase is made up of five subunits (α_2, β′, β, ω, where α is present as a dimer) and can synthesize RNA in a nonspecific manner. The holoenzyme is made up of the core polymerase subunits plus a σ (sigma) subunit. The sigma subunit is responsible for specific promoter recognition and initiation of transcription. There are seven known sigma subunits in *E. coli*, and although they share some sequence similarity, they recognize different promoter sequences and thus regulate different classes of genes.

Large amounts of highly pure RNA polymerase are necessary to study in vitro transcription and sigma interaction with core RNA polymerase. The development of polyol-responsive monoclonal antibodies (PR-mAbs),[1] which bind antigen with high affinity but dissociate in the presence of various combinations of nonchaotropic salts and polyhydroxylated compounds (polyols), have made possible the gentle purification of large, labile proteins and protein complexes under nondenaturing conditions using immunoaffinity chromatography ([1–4], for review

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[1] *Abbreviations used:* PR-mAbs, polyol-responsive monoclonal antibodies; ScFv, single-chain variable fragment; V_H, heavy chain; V_L, light chain; IMAC, immobilized metal affinity chromatography; ELISA, enzyme-linked immunosorbent assay; TCEP, Tris(2-carboxyethyl)phosphine, OPD, *ortho*-phenylenediamine; GuHCl, guanidine hydrochloride; DTT, dithiothreitol; Ni–NTA, Ni^{2+}–nitrilotriacetic acid; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; RPAS, recombinant phage antibody system.

see [5]). The isolation of the PR-mAb NT73 [6], which binds to the β′ subunit of *E. coli* RNA polymerase, has allowed the rapid purification of biologically active core RNA polymerase and holoenzyme using immunoaffinity chromatography. The epitope of NT73 on RNA polymerase has recently been determined [7]. It was shown that the 13-amino acid epitope could be fused to either the N- or C-terminus of GFP, and the PR-mAb NT73 could bind the tagged protein while immobilized on a column. Biologically active GFP could then be eluted from the column using nondenaturing combinations of a nonchaotropic salt and a polyol. This epitope tag, which was designated Softag1, can be generally applied to the purification of biologically active proteins during immunoaffinity chromatography.

Monoclonal antibodies (mAbs) have high specificity for a single epitope on the antigen and can have high affinity. These characteristics have made mAb candidates for many applications, although there are some obstacles. Hybridoma cells are fusions of two types of cells, immunized spleen cells and plasmacytoma cells, and thus they have twice the amount of genetic information. This makes the genetic material contained in hybridomas unstable, and over time they are likely to dispose of unessential genetic information, including the immunoglobulin-producing genes. Also, the current method of production via hybridoma technology is expensive and time-consuming, making it a concern for some applications.

To overcome some of these obstacles, a mAb can be produced by expressing only its Fv-fragments. The variable regions of the heavy chain (V_H) and light chain (V_L) of the immunoglobulin, connected by a 15-amino acid $(Gly_4Ser)_3$ "linker" sequence, are the most common construct. These single-chain variable fragment antibodies (scFv) are approximately one-sixth the size of full-length antibodies and are often able to maintain the high affinity and specificity of the parental mAb. scFvs are attractive for use in larger scale applications for the following reasons: if the V_H and V_L segments of the mAb gene are obtained, the maintenance of hybridoma lines would not be necessary; there would be an unlimited supply of scFv through production in bacterial culture; and it is more cost efficient to maintain bacteria than mice. These considerations make scFvs attractive alternatives to mAbs.

Our laboratory has developed PR-mAb systems for multiple proteins [5]. To circumvent the need to use mouse ascites fluid in the production of large amounts of PR-mAbs, single-chain versions of the PR-mAbs that could be expressed and purified from bacteria were worth pursuing. The PR-mAb NT73 was used as the model antibody because it is a useful and very well-characterized antibody. It was important to determine if a PR-mAb could be converted to a scFv and to verify that the specificity, affinity, and polyol-responsiveness of the parent PR-mAb were maintained when converted to a scFv.

Materials and methods

Reagents and buffers

All pH values and conductivity readings were determined at 23 °C. Ampicillin, guanidine hydrochloride (GuHCl), and dithiothreitol (DTT) were obtained from Invitrogen (Carlsbad, CA). Pristane, chloramphenicol, lysozyme, Tris(2-carboxyethyl)phosphine (TCEP), and *ortho*-phenylenediamine (OPD) were purchased from Sigma (St. Louis, MO). Luria–Bertani (LB) medium and 2× YT agar were made using various amounts of bacto-tryptone (BD Biosciences, San Jose, CA), yeast extract (DIFCO, San Jose, CA), and sodium chloride (NaCl, Sigma). Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Roche (Indianapolis, IN). Magnesium chloride ($MgCl_2$), 2-(4-morpholino)ethanesulfonic acid (MES), concentrated hydrochloric acid (HCl) and sulfuric acid (H_2SO_4), and D-glucose (dextrose) were obtained from Fisher Scientific (Fair Lawn, NJ). Tris was obtained from Amresco (Solon, OH). Surfact-Amps 20 (Tween 20) was purchased from Pierce (Rockford, IL). One percentage BLOTTO contained 1% nonfat dry milk in phosphate-buffered saline (PBS, Gibco, Grand Island, NY). Hydrogen peroxide was from Mallinckrodt Laboratory Chemicals (H_2O_2, Phillipsburg, NJ). Ni^{2+}–nitrilotriacetic acid (Ni–NTA) agarose resin was obtained from Qiagen (Valencia, CA). Enhanced chemiluminescence (ECL) Western blotting reagents were purchased from GE Healthcare (Piscataway, NJ).

Unless otherwise stated, the horseradish peroxidase (HRP)-conjugated anti-E-tag monoclonal antibody (mAb, GE Healthcare) and HRP-conjugated goat anti-mouse IgG mAb (American Qualex, La Mirada, CA) were used at 1:1000 dilution in 1% BLOTTO.

Production of the polyol-responsive monoclonal antibody NT73

The isolation and characterization of PR-mAb NT73 has been previously described [6]. Briefly, a hybridoma cell line was produced by fusing SP2/0 plasmacytoma cells and spleen cells isolated from a Balb/c ByJ mouse (Jackson Laboratories, Bar Harbor, ME) that had been previously injected with core *E. coli* RNA polymerase. Ascites fluid was produced by injecting the hybridoma cells into a Pristane-primed Balb/c ByJ mouse.

Construction of the scFv NT73 antibody

Total RNA was purified from the NT73-producing hybridoma cells using the RNeasy Protect Mini Kit (Qiagen), and the mRNA was subsequently purified using the Oligotex mRNA Mini Kit (Qiagen). The recombinant phage antibody system (RPAS) mouse scFv module (GE Healthcare) was then used to generate the scFv gene (Figs. 1A and B). For first strand cDNA synthesis of the V_H and V_L chains, primers and reverse transcriptase enzyme

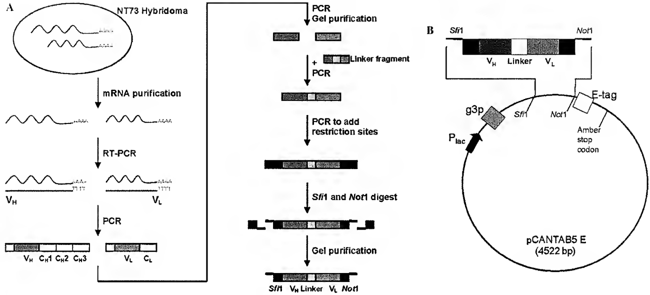


Fig. 1. Schematic representation of the RPAS module (adapted from GE Healthcare, Piscataway, NJ). (A) NT73-producing hybridoma cells were lysed and total RNA was isolated (not shown). Total mRNA was then purified over a poly(dT) affinity column. Specific primers to the variable heavy (V_H) and light (V_L) chains were used in reverse transcriptase polymerase chain reaction (RT-PCR) to produce V_H and V_L cDNA. PCR was used to amplify the cDNA products, which were then purified from an agarose gel. PCR was performed with a nucleotide linker fragment and purified V_H and V_L cDNA to produce the scFv gene. PCR was used to add restriction sites to the 5′ and 3′ end of the gene. (B) The scFv gene and pCANTAB5 E plasmid were digested with *Sfi*I and *Not*I restriction enzymes and ligated using T4 DNA ligase.

were provided with the RPAS module. For the primary polymerase chain reaction (PCR) amplification of the V_H and V_L chain cDNA, heavy chain and light chain primers and AmpliTaq DNA polymerase from the RPAS module were used. V_H and V_L chain PCR products were then purified using the Gel Extraction Kit (Qiagen) and quantified using a spectrophotometer. The V_H and V_L chain genes were joined as a single-chain by linker DNA in the orientation of 5′–V_H–Linker–V_L–3′; the linker primers and AmpliTaq DNA polymerase were provided in the RPAS module. The assembled single-chain PCR product was further amplified, and *Sfi*I and *Not*I restriction sites were added to the 5' end of the V_H chain and 3′ end of the V_L chain sequences, respectively. The scFv sequence with restriction sites was gel-purified and quantified using a spectrophotometer. The scFv fragment was digested with *Sfi*I and *Not*I restriction enzymes (New England BioLabs, Beverly, MA) and purified using gel extraction. The scFv NT73 gene was cloned into the predigested pCANTAB5 E vector using the Rapid DNA Ligation kit from Roche and sequenced at the McArdle Laboratory DNA Sequencing facility using ABI PRISM 373 DNA Sequencer (Applied Biosystems, Foster City, CA).

Colony lifts

Constructs containing scFv NT73 were transformed into competent *E. coli* (strain TG1), and the transformants were screened for specific scFv production by the colony-lift method described by the RPAS module manufacturer. Briefly, colonies were grown overnight at 30 °C on 2× YT agar containing 2% glucose and 100 µg ampicillin/ml (2× YT-GA). The colonies were then lifted onto a 0.45 µm nitrocellulose filter. The filter was placed on a second nitrocellulose filter that had been treated with 4 ml of 1 µg *E. coli* core RNA polymerase/ml for 1 h and then blocked overnight in 1% BLOTTO at 4 °C. The two filters were then placed colony-side up on a 2× YT plate containing 1 mM IPTG and 100 µg ampicillin/ml (2× YT-IA). The plates were incubated right-side up overnight at 30 °C. The filter containing the colonies was removed and transferred to a sterile petri dish. The filter that had been coated with the RNA polymerase was incubated with a 1:2000 dilution of the HRP-conjugated anti-E-tag mAb for 1 h in 1% BLOTTO. The filter was then washed three times with TBST (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20), reacted with 6 ml of the ECL reagents for 5 min, and then exposed to X-ray film for 15–60 s.

Membrane-over-agar

The procedure used was similar to the membrane-over-agar method reported for the production of staphylococcal enterotoxin for serotyping [8]. Spectropore dialysis membrane (6–8000 molecular weight cut off) was cut to the approximate size of a 100 mm petri plate, washed by boiling in water, and autoclaved. One piece was laid on the surface of each 2× YT-IA plate. Competent TG1 *E. coli* cells transformed with the scFv NT73-containing pCANTAB5 E vector were cultured overnight under suppression conditions

(30 °C) on the surface of a 2× YT-GA plate. The culture was used to inoculate the surface of the membrane, and the membrane-over-agar culture was incubated right-side up overnight at 30 °C. This allowed the accumulation of soluble antibody in the periplasm as well as secretion into the media. LB broth (5 ml) was used to flood each plate, and the culture was collected and centrifuged. The supernatant fluid was used as the source of scFv NT73 for determining specificity of the scFv.

Construction of overexpression plasmids

The scFv NT73 sequence in the pCANTAB5 E phagemid vector and *pfu* DNA polymerase were used for standard PCR reactions. The following primers were used to make a scFv with a C-terminal His-tag: 5′-GGAAT TCcatatgGCCCAGGTCCAACTG-3′ and 5′-CCCctcgag CCCTGCGGCACGCGGTTCCAG-3′. Lowercase letters represent restriction endonuclease recognition sites. All oligonucleotides were synthesized by the University of Wisconsin Biotechnology Center.

The pT7Blue-2 Blunt Cloning Kit (Novagen, Madison, WI) was used to rescue the PCR products, which allowed for blue/white colony selection. Plasmid DNA was isolated from the colonies containing the scFv insert. The pET vector pET22b(+) (Novagen) was digested with appropriate restriction enzymes and ligated with the scFv insert using the Rapid DNA Ligation kit. The ligated plasmid was then transformed into DH5α *E. coli*, purified using Mini-Prep kits (Qiagen), and verified by sequencing.

Expression of scFv NT73 antibody in E. coli hosts

For the His-tagged scFv production in BL21(DE3) pLysS or Rosetta(DE3)pLysS (Novagen), cells were grown in 1 L LB plus 100 μg ampicillin and 35 μg chloramphenicol/ml in a 2 L flask with shaking at 37 °C until the OD (600 nm) was 0.6. Then scFv production was induced by the addition of 1 mM IPTG and the cells were allowed to incubate at 37 °C for 4 h with shaking. In all cases, cells were harvested and the pellets were frozen at −20 °C for storage.

Isolation of scFv NT73 antibody inclusion bodies

Cell pellets (3.3 g wet weight) were thawed on ice, suspended in 27 ml MDN buffer (50 mM MES, pH 6.5, 0.5 mM DTT, and 100 mM NaCl), and lysed by sonication (6 times at 30 s intervals on ice). Triton X-100 (3 ml) was then added to the lysed cells and the solution was incubated on ice for 10 min, then centrifuged at 15,000*g* for 15 min. The cell pellet was again suspended in 30 ml MDN buffer containing 1% Triton X-100, incubated on ice for 10 min, and centrifuged at 15,000*g* for 15 min. The cell pellet was then washed once with 30 ml MTN buffer (50 mM MES, pH 6.5, 0.5 mM TCEP, and 100 mM NaCl) and centrifuged at 15,000*g* for 15 min. The inclusion body pellets were stored at −20 °C.

Optimization of scFv NT73 antibody inclusion body refolding

An inclusion body pellet was initially suspended in a 20 ml denaturing buffer (50 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 100 mM NaCl, 0.1 mM TCEP, and 6 M GuHCl), but then diluted with denaturing buffer so that the protein concentration was about 1 mg/ml. All protein concentrations were determined by Bradford assay using Pierce reagents and bovine serum albumin as the protein standard. Lindbro 96-well tissue culture plates were blocked with 1% BLOTTO. After removing the blocking agent, 10 μl of denatured scFv-containing inclusion body was added to 190 μl of refolding buffers #1–16 from the FoldIt protein refolding kit (Hampton Research, Aliso Viejo, CA), plus or minus oxidized/reduced glutathione (GSSG/GSH, 0.1 mM/1 mM, respectively). The final protein concentration was therefore about 0.05 mg/ml. The plate was tapped periodically but also left on a rotating platform to mix at 23 °C. The turbidity of the sample wells was measured using the 405 nm UV filter every 30 min for 4 h and then placed at 4 °C until the next time-point reading at 24 h. Immediately after optimizing inclusion body refolding conditions, the activity of refolded scFv NT73 was assayed by ELISA.

scFv NT73 antibody inclusion body refolding and purification

Refolding and purification steps were performed at 4 °C. A Gilson Minipuls 2 peristaltic pump was used for drip refolding of the scFv antibody. Inclusion body pellets (from 3.3 g wet weight cells) were thawed on ice, dissolved in 20 ml MTN buffer plus 6 M GuHCl, and centrifuged for 15 min at 15,000*g* to remove any insoluble material. The protein concentration of the supernatant was determined and diluted with MTN buffer plus 6 M GuHCl to 1 mg protein/ml. The supernatant was drip-diluted 20-fold into refolding buffer (50 mM MES, pH 6.5, 10 mM NaCl, 550 mM GuHCl, 2 mM $MgCl_2$, and 0.025% Tween 20) at a rate of 0.4 ml supernatant/min and then slowly mixed with a magnetic-stir bar for 15 min. The solution was centrifuged for 10 min at 15,000*g* to remove any aggregated material. Ni–NTA agarose resin (1 ml packed resin/10 ml supernatant) was added to the refolded solution and allowed to mix for 1 h. The solution was centrifuged for 5 min at 125*g* to gently pellet the Ni–NTA agarose resin. The supernatant was decanted and the resin was added to a column. The resin was washed twice with 10 column volumes of wash buffer (50 mM MES, pH 6.5, 500 mM NaCl, 2 mM $MgCl_2$, 0.025% Tween 20, and 20 mM imidazole). The scFv was eluted from the column using wash buffer containing 500 mM imidazole, which was added and collected one column volume at a time. Peak fractions were determined and pooled.

ELISA

The Lindbro 96-well tissue culture plate from the refolding experiment was centrifuged at 2500 rpm (Beckman TJ-6 centrifuge equipped with microplate adapter) for 15 min to

remove any aggregated protein. A multi-channel pipette was used to directly transfer 10 µl of the refolded scFv in the 16 different FoldIt kit solutions to a Lindbro 96-well tissue culture plate containing 40 µl of 1% BLOTTO per well, and which had been previously coated with core RNA polymerase (100 ng/well) and blocked with 1% BLOTTO. The final protein concentration was therefore 0.01 mg/ml. The scFv was allowed to react for 16 h at 4 °C. Unbound scFv was removed with three PBST washes (PBS plus 0.1% Tween 20), and then HRP-conjugated anti-E-tag mAb was added and allowed to incubate at room temperature for 1 h. Unbound secondary antibody was removed by five PBST washes and then 100 µl substrate (0.45 mg/ml *ortho*-phenylenediamine (OPD) and 0.03% H_2O_2 contained in 0.05 M citrate buffer, pH 5.0) was added. Reactions were quenched with 50 µl of 1 M H_2SO_4 and read at 490 nm. To determine the effect a buffer component had on the overall refolding and activity of the scFv (Table 1), the difference between the sum of the ELISA readings with (Yes) and the sum without (No) a component was determined, and that difference was divided by eight. This gave the overall effect (main effect) of the component on refolding and activity of the scFv.

ELISA-elution assay

ELISA-elution assays were performed as previously described [6,9,10]. Briefly, Lindbro 96-well tissue culture plates were coated with 100 ng/well core RNA polymerase and then blocked with 1% BLOTTO. The primary antibody, either scFv NT73 or mAb NT73 diluted in 1% BLOTTO, was allowed to react with the immobilized antigen for 1 h at room temperature. Excess antibody was removed with three washes of PBST. Either a polyol/salt buffer (50 mM MES, pH 6.5, 0.7 M NaCl, and 40% propylene glycol) or MES buffer was then added and allowed to incubate at room temperature for 20 min. Dissociated antibody and excess buffer were washed away with PBST. HRP-labeled anti-E-tag mAb or HRP-labeled goat anti-mouse IgG was allowed to react for 1 h at room temperature. Excess secondary antibody was removed with eight washes of PBST. The substrate, 100 µl of 0.45 mg/ml OPD, and 0.03% H_2O_2 contained in 0.05 M citrate buffer, pH 5.0, was added and the reaction was quenched after ~5 min using 50 µl of 1 M H_2SO_4. Samples were read at 490 nm for detection of remaining secondary antibody. An antibody, either single-chain or monoclonal, was considered polyol-responsive (it dissociated in the presence of a polyol and salt buffer) when the signal strength at 490 nm decreased at least 50% in the wells washed with the polyol/salt buffer as compared to the control MES buffer.

SDS–PAGE and Western blot

Samples were incubated at 90 °C for 4 min in sample loading buffer (0.25 M Tris–HCl, pH 6.8, 5% glycerol, 5% 2-mercaptoethanol, 3% SDS, and 0.2 mg/ml bromphenol

Table 1
Summary of results from scFv NT73 refolding optimization assay

Buffer No.	Turbidity at 24 h (405 nm)	ELISA (490 nm)	55 mM Tris, pH 8.2?	55 mM MES, pH 6.5?	Low salt? 11 mM NaCl and 0.44 mM KCl	0.055% PEG?	550 mM GuHCl?	2.2 mM Mg^{2+}/2.2 mM Ca^{2+}?	1.1 mM EDTA?	440 mM sucrose?	550 mM arginine?	Sucrose/arginine?	Reduced/oxidized glutathione?
1	0.072	0.165	Yes	No	No	Yes	No	No	Yes	No	No	No	
2	0.070	1.16	No	Yes	Yes	No	Yes	Yes	No	No	No	No	
3	0.077	0.710	No	Yes	Yes	Yes	Yes	No	Yes	No	No	Yes	
4	0.080	0.192	Yes	No	No	No	No	Yes	No	No	No	Yes	
5	0.074	0.151	No	Yes	No	No	No	Yes	No	Yes	No	No	
6	0.073	0.106	Yes	No	Yes	Yes	Yes	No	Yes	Yes	No	No	
7	0.075	0.084	Yes	No	Yes	No	Yes	Yes	No	No	Yes	No	
8	0.074	0.092	No	Yes	No	Yes	No	No	Yes	No	Yes	No	
9	0.078	1.13	No	Yes	No	Yes	Yes	Yes	No	Yes	No	No	
10	0.078	0.111	Yes	No	Yes	No	No	No	Yes	Yes	No	No	
11	0.081	0.258	Yes	No	Yes	Yes	No	Yes	No	No	Yes	No	
12	0.081	0.328	No	Yes	No	No	Yes	No	Yes	No	Yes	No	
13	0.071	0.238	Yes	No	No	No	Yes	No	Yes	No	No	No	
14	0.173	0.084	No	Yes	Yes	Yes	No	Yes	No	No	No	No	
15	0.079	0.424	No	Yes	Yes	No	No	No	Yes	No	No	Yes	
16	0.100	0.285	Yes	No	No	Yes	Yes	Yes	No	No	No	Yes	
Sum Yes			1.44	4.07	2.93	2.83	4.04	3.34	2.22	1.50	0.762	1.61	
Sum No			4.07	1.44	2.58	2.69	2.17	2.17	3.34	4.02	4.75	3.90	
Main effect:			−0.33	+0.33	+0.044	+0.018	+0.23	+0.15	−0.14	−0.32	−0.50	−0.29	No
Main effect:			−0.35	+0.35	+0.12	−0.13	+0.24	+0.087	−0.087	−0.36	−0.47	−0.25	Yes

blue) and then separated by SDS–PAGE using 12% Bis–Tris NuPAGE polyacrylamide gels (Invitrogen). For Western blots, the proteins separated by electrophoresis were transferred to 0.45 μm nitrocellulose and blocked overnight in 1% BLOTTO at 4 °C. The primary antibody was diluted into 1% BLOTTO and incubated with the blot for 1 h at room temperature. The blot was washed three times with TBST. A HRP-labeled secondary antibody was then added and incubated for 1 h at room temperature. HRP-labeled anti-E-tag mAb was used to detect scFv NT73; HRP-labeled goat anti-mouse IgG mAb was used to detect mAb NT73. The blot was washed five times with TBST, reacted with ECL Western blotting reagents for 2 min, and then exposed to X-ray film for 15–60 s.

Results and discussion

Isolation and characterization of scFv NT73

mRNA isolated from PR-mAb NT73-producing hybridoma cells was used to amplify and assemble the scFv using the RPAS mouse scFv module (Fig. 1A). This construct was then cloned into the pCANTAB5 E vector, placing the expression under the control of the *lac* promoter. A g3p secretion sequence was fused to the N-terminus and an epitope tag (E-tag) was fused to the C-terminus of the scFv (Fig. 1B). TG1 cells transformed with the construct were selected for specific scFv production by the colony-lift assay; of 48 ampicillin-resistant colonies tested, two appeared to produce RNA polymerase-reactive antibody. One of the isolates was cultured by the membrane-over-agar method to yield sufficient amounts of soluble scFv for Western blotting.

To ensure specificity, Western blots were performed (Fig. 2). Purified *E. coli* RNA polymerase, *Bacillus subtilis* RNA polymerase, and Softag1-tagged GFP samples were separated by SDS–PAGE (Fig. 2A) and transferred to nitrocellulose membrane, which was then probed with either secreted scFv from the membrane-over-agar (Fig. 2B) or mAb NT73 (Fig. 2C). *B. subtilis* RNA polymerase does not react with mAb NT73 [6]. The scFv specifically reacted with the β′ subunit of *E. coli* RNA polymerase and epitope-tagged GFP but not with *B. subtilis* RNA polymerase, which is in agreement with mAb NT73 specificity.

Expression of scFv NT73

Even though the scFv could be expressed from the pCANTAB5 E plasmid, we did not intend to use the scFv in phage-display. We instead attempted to clone it into a more robust bacterial expression system, such as the T7 promoter-driven pET expression system (Novagen). To incorporate the E-tag into the new constructs, the scFv was again amplified from the pCANTAB5 E vector by PCR to add appropriate restriction endonuclease sites and then recloned into several expression vectors, including pET28b(+) (data not shown) and pET22b(+) (Fig. 3A). This also allowed for the addition of a hexahistidine tag (His-tag) to the N-terminus, or the addition of a His-tag to the C-terminus, respectively. The scFv was sequenced

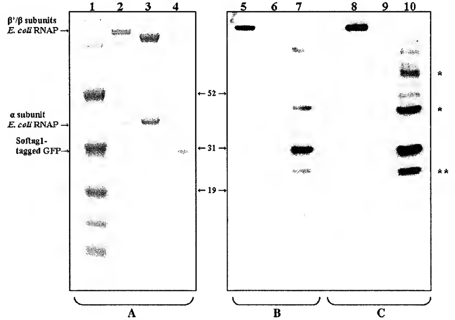
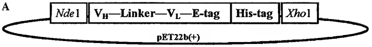


Fig. 2. SDS–PAGE and Western blot analysis of the binding specificity of scFv NT73. (A) Coomassie stained gel. (B) Western blot using scFv NT73 (undiluted supernatant from membrane-over-agar) as primary antibody and the anti-E-tag mAb as the secondary antibody. (C) Western blot using mAb NT73 (1:1000 dilution) as the primary antibody and an anti mouse-IgG mAb as the secondary antibody. Lane 1, MultiMark protein marker (in kDa); lanes 2, 5, and 8 are *E. coli* core RNAP; lanes 3, 6, and 9 are *B. subtilis* core RNAP; lanes 4, 7, and 10 are Softag1-tagged GFP. Asterisks represent either multimers (*) or breakdown products (**) of Softag1-tagged GFP.

A



B

1	ATG	GCC	CAG	GTC	CAA	CTG	CAG	GAG	TCA	GGG	GGA	GGC	TTA	GTG	AAG	CCT	GGA	GGG	TCC	CTG
1	met	ala	gln	vel	gln	leu	gln	glu	ser	gly	gly	gly	leu	vel	lys	pro	gly	gly	ser	leu
61	AAA	CTC	TCC	TGT	GCA	GCC	TCT	GGA	TTC	ACT	TTC	AGT	GGC	TAT	GCC	ATG	TCT	TGG	GTT	CGC
21	lys	leu	ser	cys	ala	ala	ser	gly	phe	thr	phe	ser	gly	tyr	ala	met	ser	trp	val	arg
																CDR1 (V_H)				
121	CAG	ACT	CCG	GAG	AAG	AGG	CTG	GAG	TGG	GTC	GCA	TCC	ATT	AGT	AGT	GGT	GGT	GGC	ACC	TAC
41	gln	thr	pro	glu	lys	erg	leu	glu	trp	val	ala	ser	ile	ser	ser	gly	gly	gly	thr	tyr
181	TAT	CCA	GAC	AGT	GTG	AAG	GGC	CGA	TTC	ACC	ATT	TTC	AGA	GAT	AAT	GCC	AGG	AAC	ATC	CTG
61	tyr	pro	esp	ser	val	lys	gly	erg	phe	thr	ile	phe	arg	esp	asn	ala	erg	esn	ile	leu
							CDR2 (V_H)													
241	TAC	CTG	CAA	ATG	AGC	AGT	CTG	AGG	TCT	GAG	GAC	ACG	GCC	ATG	TAT	TAC	TGT	GTA	AGG	GGC
81	tyr	leu	gln	met	ser	ser	leu	erg	ser	glu	asp	thr	ala	met	tyr	tyr	cys	vel	erg	gly
301	CAA	GAC	TAC	TGG	GGC	CAA	GGG	ACC	ACG	GTC	ACC	GTC	TCC	TCA	GGT	GGA	GGC	GGT	TCA	GGC
101	gln	esp	tyr	trp	gly	gln	gly	thr	thr	val	thr	val	ser	ser	gly	gly	gly	gly	ser	gly
	CDR3 (V_H)																			
361	GGA	GGT	GGC	TCT	GGC	GGT	GGC	GGA	TCG	GAC	ATC	GAG	CTC	ACT	CAG	TCT	CCA	CTC	TCC	CTG
121	gly	gly	gly	ser	gly	gly	gly	gly	ser	asp	ile	glu	leu	thr	gln	ser	pro	leu	ser	leu
		linker																		
421	CCT	GTC	AGT	CTT	GGA	GAT	CAA	GCC	TCC	ATC	TCT	TGC	AGA	TCT	AGT	CAG	AGC	CTT	GTA	CAC
141	pro	vel	ser	leu	gly	esp	gln	ela	ser	Ile	ser	cys	arg	ser	ser	gln	ser	leu	vel	his
																				CDR1 (V_L)
481	AGT	AAT	GGA	AAC	ACC	TAT	TTA	CAT	TGG	TAC	CTG	CAG	AAG	CCA	GGC	CAG	TCT	CCA	AAG	CTC
181	ser	esn	gly	esn	thr	tyr	leu	his	trp	tyr	leu	gln	lys	pro	gly	gln	ser	pro	lys	leu
541	CTG	ATC	TAC	AAA	GTT	TCC	AAC	CGA	TTT	TCT	GGG	GTC	CCA	GAC	AGG	TTC	AGT	GGC	AGT	GGA
181	leu	ile	tyr	lys	vel	ser	esn	erg	phe	ser	gly	val	pro	asp	arg	phe	ser	gly	ser	gly
								CDR2 (V_L)												
601	TCA	GGG	ACA	GAT	TTC	ACA	CTC	AAG	ATC	AGC	AGA	GTG	GAG	GCT	GAG	GAT	CTG	GGA	GTT	TAT
201	ser	gly	thr	asp	phe	thr	leu	lys	ile	ser	arg	val	glu	ala	glu	esp	leu	gly	val	tyr
661	TTC	TGC	TCT	CAA	AGT	ACA	CAT	GTT	CCG	TGG	ACG	CTC	GGT	GGA	GGG	ACC	AAG	CTG	GAA	ATA
221	phe	cys	ser	gln	ser	thr	his	val	pro	trp	thr	leu	gly	gly	gly	thr	lys	leu	glu	ile
								CDR3 (V_L)												
721	AAA	CGG	GCG	GCC	GCA	GGT	GCG	CCG	GTG	CCG	TAT	CCG	GAT	CCG	CTG	GAA	CCG	CGT	GCC	GCA
241	lys	erg	ale	ale	ale	gly	ala	pro	val	pro	tyr	pro	esp	pro	leu	glu	pro	arg	ale	ela
												E-tag								
761	GGG	CTC	GAG	CAC	CAC	CAC	CAC	CAC	CAC	TGA										
261	gly	leu	glu	his	his	his	his	his	his	Stop										
							His-tag													

Fig. 3. The sequence of His-tagged scFv NT73. (A) Diagram of the His-tagged scFv overexpression plasmid. The scFv gene in the pCANTAB5 E plasmid was amplified by PCR to add the *Nde*I and *Xho*I restriction sites to the 5′ and 3′ end, respectively. It was then inserted into the pT7Blue-2 vector and transformed into NovaBlue competent cells to allow for blue/white colony selection. The pET vector and the scFv gene in the pT7Blue-2 vector were digested with *Nde*I and *Xho*I restriction enzymes and ligated using T4 DNA ligase to produce the overexpression plasmid. (B) Nucleotide and amino acid sequences of the scFv gene as the His-tagged construct. Predicted CDRs were determined as previously described in [11]. The monoclonal antibody NT73 has an IgG_1 isotype, and the light chains are κ chains. The scFv gene is 269 amino acids total: the V_H domain is 114 amino acids; the linker sequence, $(Gly_4Ser)_3$, is 15 amino acids; the V_L domain is 113 amino acids; the E-tag is 13 amino acids preceded by the 3-amino acid $(Ala)_3$ linker sequence; and the His-tag is 6 amino acids preceded by a 5-amino acid linker sequence.

(Fig. 3B), and the conserved heavy and light chain sequences were identified, allowing the determination of the six complementarity-determining regions (CDRs) [11]. The nucleotide and amino acid sequences of scFv NT73 (excluding E- and His-tags) were submitted to GenBank and the Accession No. is DQ157432.

The selection of a proper expression host strain needed to be considered. Two common expression hosts, which each contain λ lysogens carrying T7 RNA polymerase, are BL21(DE3) and Rosetta(DE3) (Novagen). Each of these hosts can also come with an extra plasmid containing the T7 lysozyme gene, pLysS, which encodes an inhibitor of T7 RNA polymerase. The BL21(DE3) and Rosetta(DE3) host strains are deficient in the protease genes *ompT* and *lon* [12]. The Rosetta(DE3) host strain additionally provides specific *E. coli* codon tRNAs (AUA, AGG, AGA, CUA, CCC, GGA) and is designed to increase expression of eukaryotic proteins, which often use these codons that are rarely used in *E. coli*.

Preliminary results suggested that the scFv remains in the cytoplasm as inclusion bodies, so attempts were made to overproduce the scFv in the cytoplasm. The expression constructs were transformed into a variety of *E. coli* hosts, including BL21(DE3)pLysS (Fig. 4A) and Rosetta(DE3) pLysS (Fig. 4B). Keeping in mind that this was a mammalian protein being expressed in a bacterial system, it was likely that the scFv NT73 contained codons that are not commonly used in *E. coli*. As one example, in a normal *E. coli* cell, for every 1000 codons read, only 1.2 of them are the arginine codon, AGG. However, for every molecule of His-tagged scFv NT73 made (269 codons), there were four AGG codons, so it is likely that the cell quickly became depleted of charged tRNAs that correspond to AGG and therefore could not overproduce the scFv. Based on this observation, Rosetta(DE3)pLysS was used to try to overproduce the protein. As seen in Fig. 4, there was a large difference in the amount of overproduced scFv in Rosetta (DE3)pLysS as compared to BL21(DE3)pLysS (lane 6 vs

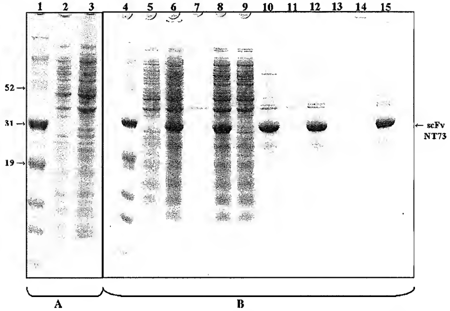


Fig. 4. SDS–PAGE analysis of the induction of His-tagged scFv NT73 in various *E. coli* strains and the purification of scFv-containing inclusion bodies from Rosetta(DE3)pLysS. (A) His-tagged scFv in BL21(DE3)pLysS. Wet weight of cell pellet was 3.0 g. (B) His-tagged scFv from Rosetta(DE3)pLysS. Wet weight of cell pellet was 3.3 g. Lanes 1 and 4 are MultiMark protein markers (in kDa); lanes 2 and 5 are whole cell lysates from uninduced cells; lanes 3 and 6 are whole cell lysates after 4 h induction with 1 mM IPTG; lane 7, LB media sample after harvesting of cells; lane 8, whole cell lysate suspended in MDN buffer with 1% Triton X-100 added after sonication; lane 9, soluble material after centrifugation of whole cell lysate; lane 10, insoluble material after centrifugation of whole cell lysate; lane 11, soluble supernatant after second MDN buffer plus 1% Triton X-100 wash; lane 12, insoluble material after second 1% Triton X-100 wash; lane 13, soluble material after MTN buffer wash; lane 14, insoluble material after suspension in 6 M GuHCl, incubation at room temperature for 30 min, and centrifugation; lane 15, material after solubilization in 6 M GuHCl, incubation, and centrifugation, which was also the starting material for refolding experiments.

lane 3, respectively). Of the strains tested, Rosetta (DE3)pLysS was best for overproduction of scFv NT73.

To isolate inclusion bodies from the whole cell lysates, washes with Triton X-100 were performed and found to be effective in removing the majority of the cell debris (Fig. 4, lanes 9 and 11) and isolating the scFv in the insoluble material (Fig. 4, lanes 10 and 12). The inclusion body pellet was solubilized in buffer containing 6 M GuHCl (Fig. 4, lane 15), and this material was used for further refolding experiments.

Refolding of scFv NT73

Initial attempts to use Tris–HCl, pH 7.4, and NaH_2PO_4, pH 7.5, buffers during the inclusion body isolation were somewhat successful. The scFv NT73 has a p*I* of 8.7; consequently, it was more likely to aggregate in buffers around its pI, including buffers such as Tris–HCl and NaH_2PO_4, which are usually used around pH 7.0–9.0.

After extensive efforts to determine favorable refolding conditions by trial-and-error, the FoldIt protein refolding kit was used to help determine the optimal refolding conditions [13]. To monitor protein refolding in the various buffers, an assay was needed to determine the "turbidity," or light scattering, of the sample. It was assumed that a more turbid sample correlated with a more aggregated or misfolded protein.

Lindbro 96-well tissue culture plates were coated with 1% BLOTTO to prevent nonspecific binding of protein. After washing away the BLOTTO, various refolding buffers were added to the wells. Solubilized His-tagged scFv NT73 inclusion body was simultaneously added to the wells containing refolding buffers to affect a 20-fold dilution of the protein concentration. Readings at 405 nm were taken every 30 min for 4 h and then again at 24 h to measure the light scattering of the solutions. A lower OD (405 nm) value indicated a less turbid sample well and was assumed to contain better refolded scFv (Table 1). The activity of the scFv was then determined by ELISA. The samples from the refolding assay were transferred to a core RNA polymerase-coated 96-well plate that had been blocked with 1% BLOTTO and used as the primary antibody of the ELISA. The anti-E-tag mAb was used as the secondary antibody. The OD (490 nm) values from the ELISA correlated with the amount of active scFv. More specifically, the higher OD (490 nm) value, the more active scFv was present (Table 1). Even though the scFv appeared refolded in some buffers, it did not necessarily mean that the scFv antibody was biologically active. For example, in Table 1, the scFv antibody appeared to be refolded in buffers 1 and 2 because the OD (405 nm) values were relatively low at 0.072 and 0.070, respectively. However, buffers 1 and 2 had very different OD (490 nm) values in the ELISA; buffer 1 had a very low value of 0.165 whereas buffer 2 had a very high value of

1.16. This suggests that between buffers 1 and 2, only in buffer 2 did the scFv antibody refold to its active state.

The results from these two assays also provided information on the effect each component of a buffer had on the overall refolding and activity of His-tagged scFv NT73. In Table 1, a larger positive value for the main effect of a buffer component meant that the factor tested was important for the proper refolding and activity of the scFv. For example, to determine if the presence of 550 mM GuHCl in the refolding buffer was important for the refolding of scFv NT73, the following equation was calculated: $[(\text{ELISA value sum of Yes}) - (\text{ELISA value sum of No})]/8 = [(4.04) - (2.17)]/8 = +0.23$. Because +0.23 is a relatively large positive value, it was concluded that the presence of GuHCl in the optimized refolding buffer was important. This statement was also true for the buffer components 55 mM MES, pH 6.5, and 2.2 mM Mg^{2+}/2.2 mM Ca^{2+}. The following buffer components had a large negative value for their main effect on refolding and were thus excluded later from the optimized refolding buffer: 55 mM Tris, pH 8.2; 440 mM sucrose; and 550 mM arginine. All other factors had either a small positive or small negative value and were considered not necessary for the refolding and activity of the scFv.

Overall, from the turbidity assay and from the ELISA, it was determined that buffer 2 plus or minus oxidized/reduced glutathione from the FoldIt protein refolding kit was the optimal refolding buffer that resulted in the most biologically active scFv NT73. Additionally, to determine if refolding of the scFv and then later forming disulfide bonds or if refolding and simultaneously forming disulfide bonds within the scFv was better, the presence or absence of 0.5 mM TCEP in the refolding buffer was tested, respectively. It was found that the presence or absence of a reducing agent did not make a difference during refolding of the scFv NT73 (data not shown).

Purification of scFv NT73

Once the scFv was refolded, it needed to be purified from minor contaminants and concentrated (Fig. 5). Ni–NTA slurry was added to the refolded scFv solution and allowed to bind to the resin. The resin was collected in a column, and the flow through was collected. As shown in Fig. 5, lane 3, the majority of the scFv was bound to the resin because it was not seen in the flow through. The resin was washed twice with buffer containing 20 mM imidazole to remove nonspecific proteins (Fig. 5, lanes 4 and 5). Based on previous high performance liquid chromatography (HPLC) experiments using an increasing imidazole concentration gradient during the elution, it was determined that the majority of scFv NT73 eluted from a Ni–NTA resin by approximately 400 mM imidazole concentration (data not shown). Consequently, a buffer containing 500 mM imidazole was used in subsequent experiments to elute scFv NT73 from a Ni–NTA resin (Fig. 5, lanes 6–9). Using the methods of drip refolding and immobilized metal affinity chromatography (IMAC) allowed the purification of approximately 60 mg of His-tagged scFv NT73 per liter of culture (Table 2).

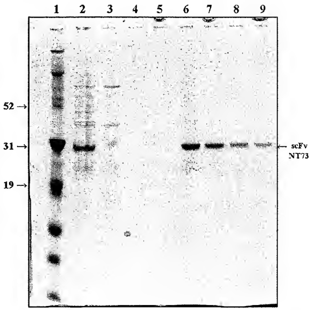


Fig. 5. SDS–PAGE analysis of the purification of His-tagged scFv NT73 over a Ni–NTA column. Lane 1, MultiMark protein marker (in kDa); lane 2, starting material- denatured scFv in buffer containing 6 M GuHCl; lane 3, flow through; lane 4, unbound material after the first wash with wash buffer; lane 5, unbound material after the second wash with wash buffer; lane 6, first peak 1-ml elution from the column using wash buffer containing 500 mM imidazole; lane 7, second 1-ml elution; lane 8, third 1-ml elution; lane 9, fourth 1-ml elution from the Ni–NTA column.

Table 2
Summary of the purification of His-tagged scFv NT73 from Rosetta(DE3)pLysS

Fraction	Total protein (mg)	scFv (mg)	Yield (%)	Purity (%)
Whole cell lysate[a]	340	~100	100	~30
Washed inclusion body	120	~100	100	~80
Ni–NTA elution peak	60	60	50	99

[a] From 1 L of culture (3.3 g wet weight).

ELISA and ELISA-elution analysis

An ELISA on a twofold dilution series of purified His-tagged scFv NT73 showed that the scFv was still able to bind to its antigen, β′ subunit of RNA polymerase (Fig. 6). Based on ELISA-elution analysis, the His-tagged scFv retains the polyol-responsiveness of its parent antibody, NT73. It releases its antigen in the presence of 0.7 M NaCl and 40% propylene glycol. Interestingly, it also releases its antigen in the presence of 40% propylene glycol alone (Fig. 7).

Immunoaffinity chromatography

The scFv NT73 could be coupled to cyanogen bromide activated Sepharose (Sigma); however, the resulting

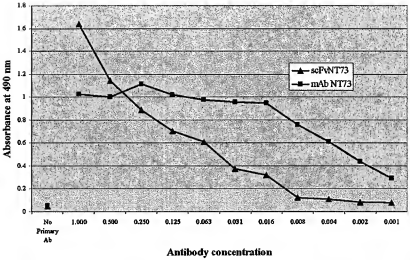


Fig. 6. ELISA of His-tagged scFv NT73 and mAb NT73. ELISA was carried out as described under Materials and methods. Antibody concentration for His-tagged scFv NT73 is in milligram per milliliter, for mAb NT73 is in microgram per milliliter.

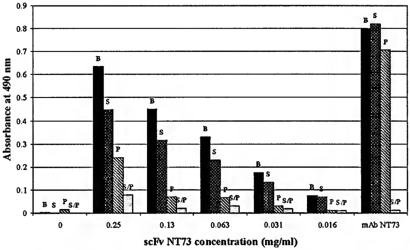


Fig. 7. ELISA-elution assay of His-tagged scFv NT73 and mAb NT73. TE buffer (B), TE buffer containing 0.75 M NaCl (S), TE buffer containing 40% propylene glycol (P), or TE buffer containing 0.75 M NaCl and 40% propylene glycol (S/P) was used in the wash step.

immunoaffinity resin was unable to pull out RNA polymerase or Softag1-tagged GFP from *E. coli* extract (data not shown). It therefore appears that the single-chain variant of NT73 has a decreased affinity for its antigen. Experiments are currently underway to determine the binding constants of PR-mAb NT73 and scFv NT73.

Conclusions

The polyol-responsive monoclonal antibody NT73 can be converted to a single-chain version. The best system for overexpression of scFv NT73 is using vector pET22b(+), which provides a C-terminal His-tag, and host Rosetta(DE3)pLysS, which provides rare codon tRNAs. The His-tagged scFv retains the polyol-responsive properties of the monoclonal antibody, NT73, but loses considerable affinity for its antigen.

It should be noted that not all scFv antibodies necessarily retain the same binding properties as the intact monoclonal antibody. For example, monoclonal antibodies were screened to bind antigen in the presence of agents that destabilize protein–protein interactions, such as DMSO, urea, guanidine hydrochloride, high salt, and extreme pH conditions, and they were also converted to scFv antibodies [14]. The binding properties of the scFv antibody were compared to the intact antibody in the presence of the destabilizing agents and were found to be significantly different. This suggested that when converted to a scFv antibody, the types and three-dimensional orientation of hydrophobic, hydrogen bonding and ionic molecular interactions

between antigen and antibody can change. This may explain the observation that scFv NT73 has reduced affinity for its antigen, the β′ subunit of RNA polymerase.

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